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**Eicosapentaenoic acid prevents salt sensitivity in diabetic rats decreasing oxidative stress**

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Running head: *Eicosapentaenoic acid decreases oxidative stress in diabetic rats*

**Abstract**

**Background:** Salt sensitivity (SS) is associated with increased cardiovascular risk in type-2 diabetes mellitus (T2-DM) patients due to an increase in renal oxidation. ω-3 polyunsaturated fatty acids have shown antioxidant effects, but a typical Western diet has limited content of them. In particular, ω-3 PUFAs are able to activate Nrf-2 (nuclear factor erythroid 2-related factor) to prevent DM-related complications by mitigating oxidative stress. Therefore, we hypothesized that eicosapentaenoic acid (EPA, ω-3) modulates SS in T2-DM rats, by decreasing renal oxidative stress via Nrf-2 activation, and enhancing the anti-inflammatory response via IL-6 modulation. **Methods:** 3-months-old male rats (n=40) were fed with a normal Na-diet (NNaD) and randomly selected into 4 groups: i. healthy Wistar rats, non-diabetic rats (Wi), ii. diabetic control (eSS), iii. arachidonic acid-treated eSS (ω-6) (AA), iv. EPA-treated eSS (ω-3) (EPA). After one year, rats were placed in metabolic cages for 7 days and fed with a NNaD and followed by a 7 days period with high Na-diet (HNaD). Systolic blood pressure (SBP), body weight (BW), serum interleukin-6 (IL-6) and reactive oxygen species (ROS) levels were determined at the end of each 7-day period. Glycated hemoglobin (HbA1c), triglycerides (TAG), creatinine and cholesterol (Chol) were determined. ROS levels and Nrf-2 expression in kidney lysates were also assayed. Histological changes were evaluated. T-test or ANOVA were used for statistical analysis. **Results:** Following HNaD, SBP increased in both control eSS and AA groups, but not in EPA and Wi groups. However, HbA1c remained unchanged by the treatments, suggesting that the beneficial effect observed was independent of HbA1c level. IL-6 levels were higher in eSS and AA, but it remained unaltered in EPA and Wi rats following HNaD diet. Interestingly, EPA protected against serum ROS in rats fed the HNaD, whereas AA did not. In kidney lysates, ROS decreased significantly in EPA compared to eSS, and consistently, Nrf-2 expression was higher compared to AA and eSS. Diabetic rats presented focal segmental sclerosis, adherence to Bowman capsule and mild to moderate interstitial fibrosis. EPA and AA treatment prevented the kidney damage. **Conclusion:** An adequate ω3:ω6 prevents SS in diabetic rats, by a mechanism independent of glucose metabolism but associated to the prevention of renal oxidative stress generation. These data suggest that EPA antioxidant properties may prevent the development of hypertension or kidney damage.

**Keywords:** Salt sensitivity, hypertension, Eicosapentaenoic acid, Type 2 diabetes mellitus, free radicals

## INTRODUCTION

Diabetes mellitus (DM) is a major cause of death and disability worldwide and is a strong risk factor for cardiovascular disease. In particular, diabetic nephropathy (DN) remains a significant problem despite efforts to limit the impact of the disease on such end-organ damage. In such a complex milieu where no single treatment can halt DN progression, it has been found that interactions among metabolic and hemodynamic factors are involved in the development of renal lesions in patients with DM (1).

Salt Sensitivity, defined by an increase of >10% of blood pressure, secondary to sodium load, is one of the initial changes observed during the development of hypertension in DM. According to the classic concept of Guyton and Coleman (2), high salt intake increases circulating volume, which leads to a rise in the renal perfusion pressure, immediately followed by increased natriuresis that restores the circulating volume. This pressure-natriuresis mechanism prevents the increase in blood pressure (BP) that could arise from a transient increase of circulating volume. Thus, the deterioration of this mechanism increases the circulating volume and blood pressure leading to hypertension (3).

Several studies reported the infiltration of macrophages and pro-inflammatory cells in the kidney at different stages of DN. The inflammatory infiltrate produces reactive oxygen species (ROS) and pro-inflammatory cytokines, which lead to upregulation of chronic systemic inflammation and mediate DN progression (4). As a consequence of inflammation, a variety of cytokines and acute phase proteins are released in order to augment or attenuate the inflammatory response. The main inflammatory cytokines involved in the development of DN are interleukin 6 (IL-6), as well as IL-1β, IL-18 and tumor necrosis factor-$\alpha$ that might contribute to the progression of renal injury either, directly or indirectly (5). Thus, chronic inflammation at the kidney tissue contributes to DN not only as a consequence of a direct effect of pro-inflammatory mediators on cellular signaling, but also by creating a state of oxidative stress, and under these conditions, sodium reabsorption is increased (6).

In recent years, substantial evidence implicating nuclear factor (erythroid derived 2)-like 2 (Nrf2), a redox-sensitive transcription factor, in inflammation and associated disorders have been provided. In this setting, the therapeutic potential of Nrf2 activation in DM as relating to the control of oxidative stress has been described (7, 8). Chronic inflammation and oxidative stress contribute not only to DN development, but also to increased sodium reabsorption and enhancing circulatory volume (6), a condition associated with abnormal pressure natriuresis. It is widely believed that abnormal pressure natriuresis is the initial abnormality observed before

the fully development of hypertension (9). In this setting, the relative contribution of inter-individual differences based on genetic background, nutrition, physical activity, and other environmental factors has not been fully elucidated.

Understanding how these factors interact is necessary to tackle the global burden of hypertension triggered by DM. In particular, the pathophysiological effects of diet have drawn attention in response to the increasing worldwide adoption of the Western diet and the accompanying rise in the rate of the incidence of obesity, an associated outcome of DM (10). In particular, high intake of ω-6 polyunsaturated fatty acids (PUFAs) and lower intake of ω-3 PUFAs, typical of a Western diet, exert several functions that play significant roles in inflammation, metabolism, and regulation of intracellular processes. In particular, supplementation of eicosapentaenoic acid (EPA 20:5, ω-3) is an important regulator of cardiovascular health by decreasing the levels of markers and mediators of inflammation such as the cytokines interleukin-1β and tumor necrosis factor α (11). Therefore, we hypothesized that nutritional supplementation with EPA 20:5, ω-3 prevents elevation of blood pressure due to sodium load in type 2 Diabetes Mellitus (T2-DM) rats by decreasing renal oxidative stress via Nrf2 activation and IL-6 release.

## METHODS

### Experimental design

eSS rats are a stable strain derived from Wistar, and are a murine model of T2-DM characterized by fasting hyperglycemia, glucose intolerance, hyperinsulinemia, and early hypertriglyceridemia. Diabetic symptoms in this model worsen with age as insulin release decreases, closely resembling type-2 DM seen in adult humans. eSS rats were kindly provided by Prof. Tarres and Martinez from the University of Rosario, Argentina (12). Three-month-old male Wistar or eSS rats (150-200 g) were randomized and housed in cages in groups of four. Animals were maintained under standard environmental conditions (12h light/dark cycles, at 20-25°C with controlled humidity) and provided the standard isocaloric chow diet and water, both *ad libitum*. Body weight was determined once a week. The study was conducted in accordance with the guidelines set forth by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All the procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals at the School of Medicine, University of Cordoba, Argentina (Protocol approval CICUAL 62/18).

### Animal treatment

3-months-old male rats (n=40) were fed with normal Na-diet (NNaD) (0.4% NaCl) (rat/mouse chow diet by GEPSA, Pilar Group, Argentina). and divided into four groups, in order to be supplemented with different polyunsaturated fatty acids by intraperitoneal injection (ip): i. Wistar rats (Wi) were used as healthy controls, ii. diabetic control (eSS), iii. eSS treated with arachidonic acid (AA, 20:4 ω-6) (2.5 mg, i.p. monthly) (AA), iv. eSS treated with EPA (EPA, 20:5 ω-3) (2.5 mg/ip monthly) (EPA). After 1-year treatment, they were placed in metabolic cages to acclimatize for 7 days, followed by a first metabolic period feeding with NNaD, and again another 7 days period, fed a high Na-diet (HNaD) (4% NaCl), which contained granular sodium chloride (JT Backer, Argentina), manually mixed with the normal sodium diet.

Systolic blood pressure (SBP, mmHg), body weight, serum interleukin-6 levels (IL-6, pg/mL) and ROS serum levels (IMF) were determined after the NNaD and HNaD periods. Besides, glycated hemoglobin (HbA1c), triglycerides (TAG), creatinine and cholesterol (Chol) (n=10) were assayed after the NNaD period. After SBP and blood determinations were done, oxidative stress was determined through the assessment of ROS levels in kidney lysates using fluorescence (MIF) in the presence of dichlorodihydrofluorescein diacetate ($DCFH_2DA$) (n=4 per

group). Besides, immunohistochemistry for Nrf2 expression was performed in fixed kidney samples (n=3 per group).

**Systolic blood pressure determination**

Systolic blood pressure (SBP) was determined at the end of the NNaD and HNaD periods using the tail-cuff method (NIBP Blood Pressure Systems, Model I229) as we reported previously (13). Briefly, in a quiet room, animals were trained on tails-cuff inflation procedures for one week before the determination of the final blood pressure. Each rat was placed in a plastic restraint maintained at 33-36°C, with its tail passing through the optical sensor and the compression cuff and then taped to the platform. The cuff was connected to a blood pressure monitor. On inflation, the cuff stopped the blood flow through the tail, and upon deflation, the sensor detected the reappearance of the blood flow. This was used as a measure of systolic blood pressure. Results were expressed as a mean ± SEM from 3 readings.

**Serum lipid, blood glucose and glycosylated hemoglobin determination**

The peripheral blood was obtained to determine postprandial glucose, HbA1c, triglycerides (TAG), creatinine and total cholesterol (Chol) levels using commercially available kits as reported previously (13). After samples were obtained, euthanasia was performed on the rats, and then were sacrificed by decapitation.

**Serum IL-6 detection**

Before and after HNaD, blood was drawn into vacutainers without anti-coagulant from the rat tail vein, from which serum was separated by centrifugation at 3000 × g for 15 minutes, and maintained at -80ºC to determine IL-6 levels (n=10). IL-6 content was analyzed using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (BD Biosciences, SACCO srl Cadorago, Italy). Standards with known amounts of IL-6 were used to convert values into absolute concentrations of IL-6 in pg/mL. IL-6 levels are shown as a mean ± SEM from 3 readings (14).

**Intracellular ROS determination in kidney and serum**

Intracellular ROS was also determined in serum as well as in kidney lysates (n=4) harvest at the end of the protocol. ROS levels were determined by the dichlorodihydrofluorescein diacetate (DCFH2DA) method. Briefly, kidney lysates were homogenized at 4°C in TBS buffer [50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% (v/v) Nonidet P-

40; 25 mM NaF; 0.5% (w/v) sodium deoxycholate; 10% (w/v) SDS; 1 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 1 mM orthovanadate; and 10 mM sodium pyrophosphate] and centrifuged at 1000 × g for 10 minutes at 4°C. The supernatant was incubated with 50 µM DCFH2DA for 30 minutes at 37 °C in dark. The fluorescence intensity was determined using a PerkinElmer luminescence spectrometer at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. To correct background fluorescence, samples were incubated under the same conditions but without fluorescent dyes. Results were normalized by protein concentration (fluorescence intensity/mg protein) and expressed relative to the control group. Protein concentrations were quantified by the Bradford assay (Bio Rad reagent) (15).

**Nrf-2 expression**

Because oxidative stress is a major pathogenic and aggravating factor for kidney diseases, the Nrf-2 expression has been proposed to be a therapeutic target for renal protection. Nrf-2 expression was evaluated by immunohistochemistry on stained slides from a 4-µm section of kidney tissue that had been fixed and embedded previously. After antigen-unmasking treatment, the sections were washed and incubated with the hamster anti-rat Nrf-2 (1:100) monoclonal antibody. After washing with a TBS buffer, the sections were placed on a chromogen diaminobenzidine reaction, giving a brown positive precipitate. Nrf-2-positive cells were counted from slides from three samples for each condition per 10 microscopic fields (20× magnification) using an Olympus BH2 light microscope in a blinded manner. Nrf-2-positive cells were expressed as a mean ± SEM of Nrf-2-positive cells/field (16).

**Histological study**

After removal, the right kidney was fixed by a 4% paraformaldehyde infusion. The tissue was then embedded in paraffin for assessment by light microscopy and immunohistochemistry. 2-3 µm sections were cut and stained with hematoxylin-eosin and periodic acid-Schiff. Glomerular damage (presence of fibrosis, adherence to the capsule, and mesangial expansion and proliferation), interstitial mononuclear cell infiltration, and arteriolar thickening were assessed. A minimum of 100 glomeruli were evaluated in each kidney. The pathologist was blind to the sample and used x40 resolution to grade the severity in a scale from 0 to 3 as follows: 0 = absent, 1 = mild, 2 = moderate, and 3 = severe. An average score was obtained for both glomerular and interstitial changes.

**Statistical Analysis**

Data are expressed as mean ± SEM of at least three independent experiments. Statistical analysis was performed using t-test (two-tailed probability value) to compare two groups or ANOVA for multiple groups using GraphPad Prism version 8.00 for Windows (San Diego, CA, USA) $p \leq 0.05$ was considered statistically significant.

**RESULTS**

**HNaD increased, whereas EPA prevented blood systolic pressure in diabetic rats**

Healthy (Wi) rats weighed 37% more than the untreated diabetic (eSS) rats of the same age; however, no difference was observed in the basal SBP. As expected, T2-DM rats showed an elevated HbA1c (+43%) compared with the Wi group, additionally the eSS group showed a higher postprandial glucose, Chol and TAG levels. All treated T2-DM treated rats had lower serum lipids compared to the eSS group. Renal functions were similar between groups. Table 1 shows the physiological parameters of each group before HNaD feeding.

Next, we evaluated the effect of sodium load on SBP. In the eSS group, HNaD increased SBP from 109±6 mmHg to 121±5 mmHg (11.2%±2.3; p≤0.01), as well as in the AA-treated group (from 120.0±4.0 mmHg to 133.0±2.0 mmHg, 10.2%±1.6, p≤0.003).

By contrast, SBP in the EPA-treated group (from 126.0±2.0 mmHg to 129.0±2.0 mmHg, 2.9%±3.8, p>0.28), and in Wi (from 115.0±6.0 to 117.0±5.0 mmHg, 1.7%±2.1, p>0.8) did not change. No difference in food intake was observed between the groups (average intake 18±1 gr/day/rat) during the metabolic studies. Thus, EPA treatment prevented the increase in SBP in the T2-DM rats after sodium load. Given these data, we got insight into the mechanism by which EPA was able to prevent the increase on SBP.

**EPA decreased serum IL-6 and ROS**

Since slow chronic inflammation may increase blood pressure, we investigated if this elevation of SBP was associated with an inflammatory mechanism. Therefore we determined serum IL-6 levels after the NNaD and HNaD period. HNaD increased IL-6 levels in eSS treated rats from 2.0±0.8 to 2.4±0.1 pg/mL and in AA from 1.6±0.1 to 2.0±0.1 pg/mL. In EPA-treated rats, as well as in healthy Wi rats, IL-6 levels were not increased after HNaD (1.3±0.1 vs 1.4±0.1 pg/mL and 1.4±0.1 vs 1.3±0.1 pg/mL, respectively) (Fig. 2).

To add further evidence that the slow chronic inflammation mechanism is involved on the increase of SBP, we determined serum ROS levels (Fig. 3b) after the NNaD and HNaD periods. Serum ROS levels in eSS rats increased with HNaD, as well as in AA-treated eSS rats. By contrast, EPA-treated animals did not show higher ROS levels after HNaD. These results indicate that EPA prevents the increase of serum IL-6 and ROS in eSS rats after sodium load.

**EPA decreased kidney ROS**

Since the kidney is the mayor effector of the pressure natriuretic mechanism, and serum changes may not represent changes on interstitial kidney, we investigated the effect of EPA treatment on ROS levels in kidney tissues. At the end of the HNaD period, ROS levels were determined in kidney lysates (Fig. 3a). EPA-treated group showed significantly less (-84.8%, p≤0.05) ROS level with respect to eSS, suggesting that during HNaD, the intrarenal ROS production is limited compared to eSS. Although the AA-treated group showed a decreased ROS levels compared to the eSS group, the ROS levels were still higher with respect to the EPA-treated rats.

**EPA enhanced Nrf-2 expression**

Nrf2 is a protein able to regulate the cell's antioxidant response against oxidative damage triggered by injury and inflammation, and it can be stimulated by several drugs. Thus we evaluated the effect of EPA supplementation on Nrf2 kidney expression after animals were loaded with high sodium diet. Fig. 4 shows Nrf2 expression in the kidney, while Table 2 and Fig. 5 show changes in kidneys from sodium-overloaded, T2-DM-bearing rats after treatment with fatty acids. In rats supplemented with EPA, the expression of Nrf-2 was higher compared to eSS (124±8.6 vs 14±1 positive cells/field) and AA treatment (4.9±0.39 positive cells/field). Nrf2 expression in Wi rats was similar to EPA.

**Histological evaluation**

To evaluate if these effects were associated with morphological changes, kidneys from all the groups were evaluated (Fig 5, Table 2). As expected, untreated diabetic rats had kidneys showing significant glomerular changes. There were focal segmental sclerosis (FSGS) and adherence to Bowman capsule. At the interstitial level, they exhibited mild to moderate interstitial fibrosis. On the other hand, after EPA and AA treatments, a significant improvement was observed, not only in the glomerular, but also on the interstitial damage. At the glomerular level, the mesangial matrix was expanded in segmental localization, and interstitial fibrosis was either absent or focal and mild. These findings suggest that EPA supplementation has an anti-inflammatory effect and is associated with morphological changes.

## DISCUSSION AND CONCLUSION

Our results have shown that EPA treatment was able to minimize inflammation as well as oxidative stress in T2-DM through Nrf2 activation during sodium load, effects that were associated with less glomerular sclerosis as well as less interstitial fibrosis. This effectively prevented the salt sensitivity observed in untreated diabetic rats, and it was independent of glucose homeostasis, since HbA1c did not change. In particular, EPA supplementation prevented the deleterious outcome by improving endothelial function, thus preventing increased blood pressure in DM after sodium load.

HNaD increased systolic blood pressure (SBP) in the eSS rats, a model of T2-DM, while EPA supplementation prevented increase in blood pressure; whereas arachidonic acid (AA) did not. Salt sensitivity (SS), according to the American Heart Association (AHA), is a trait in which blood pressure increases in response to changes in salt intake, and this salt sensitivity is “a risk factor for cardiovascular mortality and morbidity, independent of and as powerful as BP” (17).

Although the criteria for identifying salt sensitivity are not standardized, it is defined as a change in blood pressure (office measurement) of 10% or at least 5 mmHg, in response to a change in NaCl (18). Another definition of salt sensitivity is an increase in the mean arterial blood pressure (MAP) of at least 4 mmHg (24-h ambulatory blood pressure monitoring) with an increase in NaCl intake (19). A salt sensitivity index (the difference between MAP on low-salt and high-salt diets, divided by the MAP on a low-salt diet) of at least 5% is also another definition of salt sensitivity (20). However, the most reliable method to measure salt sensitivity is the blood pressure response before and after a change in dietary salt intake (21).

Hypertension and salt sensitivity are both conditions resulting from genetic predisposition, coupled with environmental influences, such as excessive sodium consumption and sedentary lifestyles. About one-third of the world's population has hypertension, which is responsible for almost 50% of deaths from stroke and coronary heart disease (22). However, these statistics do not distinguish salt-sensitive from salt-resistant hypertension or include normotensive patients who are salt-sensitive. This distinction is important because salt sensitivity, independent of blood pressure, is a risk factor for cardiovascular morbidity and mortality (23, 24) and other diseases, for example, asthma, gastric carcinoma, osteoporosis, and renal dysfunction (25). Therefore, strategies that prevent from developing salt sensitivity may be cost-effective.

Almost five decades ago, Guyton and Coleman (2) proposed salt sensitivity to be the result of kidney malfunction. However, recent studies suggest that non-osmotic salt accumulation in the skin interstitium (26) and changes in endothelial surface layer characteristics which lead to alteration of endothelial cell function (27, 28) also play an important role in nonosmotic storage of salt. Other investigators (29, 30) identified two novel pathways in salt-sensitive hypertension: the β2-adrenergic stimulant-glucocorticoid receptor (GR)-with-no-lysine kinase (WNK)4-Na+-Cl- cotransporter pathway and the Ras-related C3 botulinum toxin substrate (Rac)1-mineralocorticoid receptor pathway, both active in DCT, connecting tubules, and collecting ducts. These new concepts emphasize that sodium homeostasis and salt sensitivity seem to be related not only by kidney malfunction, but also by endothelial dysfunction.

Chronic high sodium diet induces kidney damage at least via the formation of reactive oxygen species (ROS), such as superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$) and peroxynitrite ($ONO_2^-$). They increase sodium reabsorption via a reduction of NO bioavailability (31) or by enhancing transport in a NO-independent manner via (32) PKCα activation (33). Either mechanisms increase electrolyte retention and extracellular fluid volume (6). Additionally, long term HNaD increases via ROS derivatives can increase urinary protein excretion and decrease endothelial response to acetylcholine (13) in the absence of hypertension, demonstrating that HNaD, by itself induces tissue damage.

Our results demonstrate that acute load of sodium in diabetic scenery has the unfavorable effect of at least increasing tubular oxidative stress, and consequently salt sensitivity. While this increase was not observed in animals treated with EPA (ω-3), AA supplementation did not prevent the increase of ROS or salt sensitivity. Hypertension is twice as frequent in patients with diabetes compared with those who do not have diabetes. The major cause of morbidity and mortality in diabetes is cardiovascular disease, which is exacerbated by hypertension (34). Even if the role of ω-3 fatty acids in hypertension has been widely studied, there remains scanty data on that of SS. With respect to blood pressure, several observational studies have demonstrated an association of ω-3 fatty acid intake to low blood pressure levels, and interventional studies using ω-3 fatty acid supplementation have shown blood pressure-lowering effects. Of important mention is a population-based international study of macro- and micro-nutrients and blood pressure, which surveyed 4680 men and women, aged 40–59 from 17 population samples. It found an inverse association between blood pressure and ω-3 fatty acid intake (35). A prospective and interventional study also found that the long-term use of EPA is effective for the prevention of major coronary events in hypercholesterolaemic patients in Japan, who consume a large amount of fish (36). Other studies reported both similar and opposite results. A recent meta-analysis designed by Federico Popoff et al. (37) to evaluate the effect of omega-3 fatty acids on mortality, morbidity, and adverse events in patients with acute myocardial infarction, found no benefit of omega 3 fatty-acids supplementation. The cause of this

contradictory effect may reside in the endothelial health, suggesting that patients with important endothelial disease may not respond properly.

A large body of evidence from experimental, clinical and epidemiological research has also demonstrated the potential benefits of ω-3 fatty acids, such as EPA on cardiovascular health, including anti-atherogenic, anti-arrhythmic, and plaque stability effects, as well as the improvement of endothelial/platelet function. Besides, ω-3 fatty acids are able to switch arachidonic acid-derived eicosanoid profiles and convert ω-3 fatty acids to vasodilators and platelet anti-coagulation factors (38).

Many of the underlying molecular mechanisms causing microvascular and macrovascular complications of diabetes include oxidative stress and inflammation. One of the potential immune mediators in hypertension is interleukin-6 (IL-6). Activation of the intrarenal Renin-Angiotensin Sistem (RAS) has been proposed to facilitate the development of AngII-dependent hypertension, whereas IL-6 contributes to the increase of angiotensinogen expression in cultured renal proximal tubular cells (39). Moreover, Zhang et al. (40) demonstrated that intrarenal IL-6 is associated with AngII-dependent hypertension. We found that one mechanism involved could be the modulation of pro-inflammatory IL-6, whose levels were higher in the eSS- and the AA-treated rats, but not in the EPA-treated and the Wi rats, following HNaD feeding. It has been shown that ω-3 fatty acids such as EPA, have beneficial impacts on multiple risk factors linked to T2-DM including blood pressure, as they benefit the heart and blood vessel functions, as well as the blood lipids due to their antithrombotic, anti-inflammatory and anti-oxidative actions (41). In particular, the anti-inflammatory effects of EPA are mediated through the reduction of arachidonic acid (AA)-derived inflammatory mediators, the activation of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), the G-protein-coupled receptor (GPR) 120 as an agonist (42), and stimulation of the AMPK/SIRT pathway (43). In our model, although we did not determine any EPA derivative, there exists a general agreement that EPA exhibits anti-inflammatory properties through its metabolites in each organ and that it has pleiotropic and anti-inflammatory effects on various tissues and lesions such as the atherosclerotic lesions (44).

Pro-inflammatory cytokines, such as IL-6 plays such an important role in hypertension, and oxidant generation is an inherent participant in the process of increased sodium reabsorption and increased blood pressure (6). Consistently, we also demonstrated that EPA treatment was able to protect against increases in serum ROS levels as it has also been shown by many other investigators (45, 46, 47).

In the eSS rats, ROS levels increased upon feeding on HNaD, while it decreased significantly in the EPA-treated rats compared to the eSS and the AA-treated groups. It has been demonstrated that oxidative stress-induced complications of diabetes may include stroke, neuropathy, retinopathy, and nephropathy. Elevated ROS levels in diabetes may be due to increase in the production and/or decrease in the destruction by catalase, superoxide dismutase and glutathione peroxidase antioxidants (48). In particular, it is believed that oxidative stress plays an important role in the development of vascular complications in T2-DM. Alterations in redox homeostasis through increased intake of ω-3 fatty acids have been linked to the activation of the Nrf2 pathway, by which this transcription factor, key in regulating glucose and lipid metabolism as well as redox homeostasis, induces the transcription of endogenous antioxidants (49).

We determined Nrf2 expressions in kidney. In our model, Nrf2 expression was higher in EPA-treated rats compared to the AA-treated and eSS groups, showing that Nrf2 is involved in modulating ROS. In line with our finding, the comparative metabolic effects of a diet rich in saturated fat versus a ω-3-enriched diet in a mice model that overexpresses the endogenous antioxidant catalase was recently reported. The eight weeks of dietary intervention showed that the mice overexpressing endogenous catalase, when fed an ω-3 fatty acid-enriched diet, as against a saturated fat diet, activated GPR120-Nrf2 cross-talk to maintain balanced energy metabolism, normal circadian rhythm, and insulin sensitivity, thus reducing the risk of metabolic syndrome and associated diseases compared to their wild-type controls (50). Furthermore, Nrf2 is a redox-sensitive transcription factor activated by long chain fatty acids (including EPA), phenolic antioxidants, and imbalances in redox stress. Raising levels of Nrf2 by endogenous production of electrophilic products or pharmacological agents have been shown to prevent or act as therapies for T2-DM and cardiovascular disease through activating anti-inflammatory pathways (51).

Low-grade inflammation is a common feature of kidney diseases, which usually already exists before the need to start renal replacement therapy, and evidence suggests that persistent inflammation may also be, *per se*, a risk factor for the progression of CKD and vascular disease. Many factors, including retention of pro-inflammatory cytokines, advanced glycation end products, reactive oxygen species, autonomic dysfunctions and volume overload may contribute to inflammation when renal function declines. As noted above, Nrf2 plays a central part in basal activity and coordinated induction of several genes encoding antioxidant and phase 2 detoxifying enzymes, and related proteins. Consequently, constitutive Nrf2 activity is critical for maintaining redox balance in normal conditions, and its induction in response to oxidative stress is essential for protecting against oxidative stress. However, studies conducted in animals with 5/6 nephrectomy-induced CKD have revealed that despite the presence of oxidative stress and inflammation, which should have induced Nrf2 activation and upregulation of its target genes, CKD animals exhibited a marked and time-dependent decline in nuclear Nrf2 content, pointing to its impaired activation in the remnant kidney (52). This impaired activation of Nrf2 and the expression of the antioxidant enzymes can be restored by Olmesartan, an Angiotensin II receptor blocker (53). Under these conditions, Olmesartan therapy prevented nephropathy, suppressed oxidative stress and inflammation, and restored Nrf2 activation and expression of the antioxidant enzymes.

Equally, weak Nrf2 activators commonly found in foods or dietary supplements have renoprotective effects in rodent models. For example sulforaphane, an organosulfur compound found in cruciferous vegetables has been shown to ameliorate nephropathy in animals with streptozotocin-induced diabetes (54), mice with anti-glomerular basement membrane glomerulonephritis (55), and cisplatin-induced nephrotoxicity (56).

Taken together, our data and these findings support the role of Nrf2 deficiency in the pathogenesis of oxidative stress, inflammation, and progression of CKD and the renoprotective effect of Nrf2 activators. New therapeutic approaches targeting oxidative stress and inflammation are currently being developed to treat diabetes-associated cardiovascular complications; such as the trials using bardoxolone methyl in which a

reduction of serum creatinine in human with kidney damage was observed (57). However, larger trials are needed to investigate the efficiency of this drug. These studies are in agreement with our results showing that mechanisms that activate Nrf2 expression or activity prevent the low grade inflammation process that may finally develop kidney damage.

Overall, this study provides compelling evidence that an adequate ω-3-supplementation was able to minimize inflammation as well as oxidative stress through Nrf2 activation in T2-DM following Na-overload. EPA effectively prevented the salt sensitivity observed in untreated diabetic rats, independent of glucose homeostasis since HbA1c did not change. In particular, EPA supplementation did not present any deleterious effect as it improved endothelial function, thus preventing increased blood pressure in DM after Na-load.

To date, several policies have been proposed to reduce the salt intake of populations to the level recommended by the WHO or other organizations. Some of these policies have been executed at the population-based level, including reformulation of the foods, taxation and food labeling, while some have been performed at the individual level, including dietary counseling (58, 59) to prevent the increase of blood pressure and provide a better control of the hypertensive population. Because of patient compliance to low sodium diet is low, we evaluated if supplementation with ω-3 (EPA) may help to delay the increase in blood pressure. Overall, supplementation with EPA in normotensive diabetic rats fed high-sodium diet, prevented salt sensitivity via the upregulation of Nrf2 expression.

**Authors' contribution**:

MVM and NHG conceived, coordinated the study and wrote the paper. MVM, JHM and NHG designed, performed the experiments and analyzed the result data shown. MVM, NHG, JHM, GR, and EIOA prepared the manuscript. EB and NO provided technical assistance, essential reagents and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

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**Conflict of interest**

The author states that there is no conflict of interest.

**Ethical approval**

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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**Figure Legends**

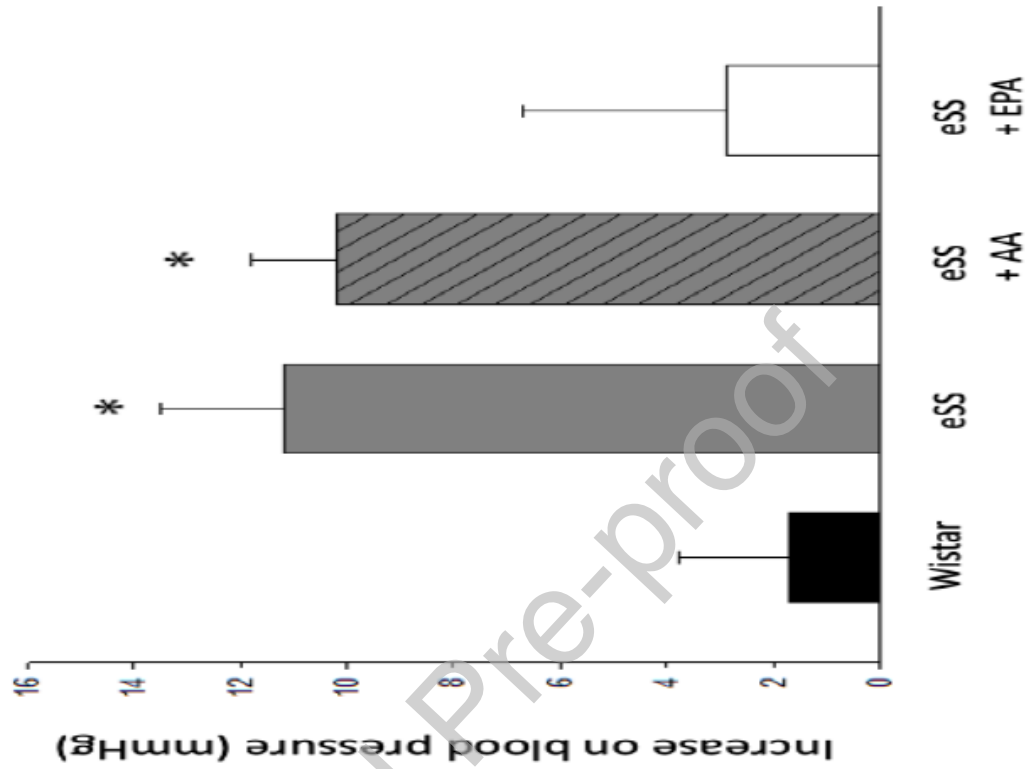



**Figure 1. Effect of High Sodium Diet on blood pressure.** After one week of HNaD, blood pressure increased only in eSS and AA-treated rats. EPA prevented increase in blood pressure. * $p \leq 0.05$ vs eSS + EPA (n=10) and Wistar rats (n=10).

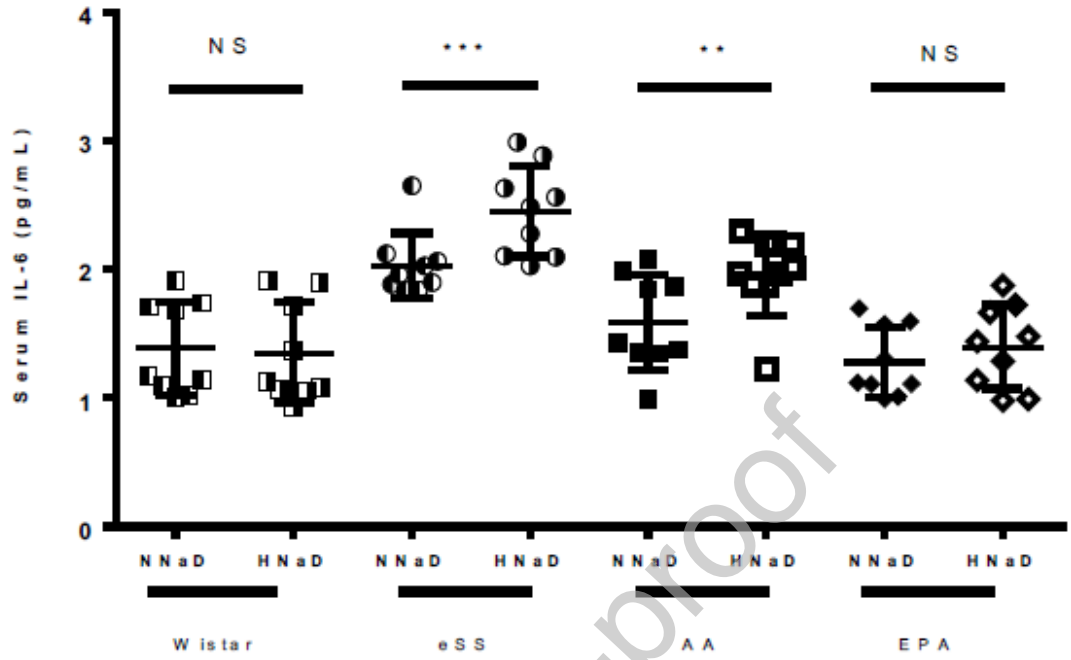
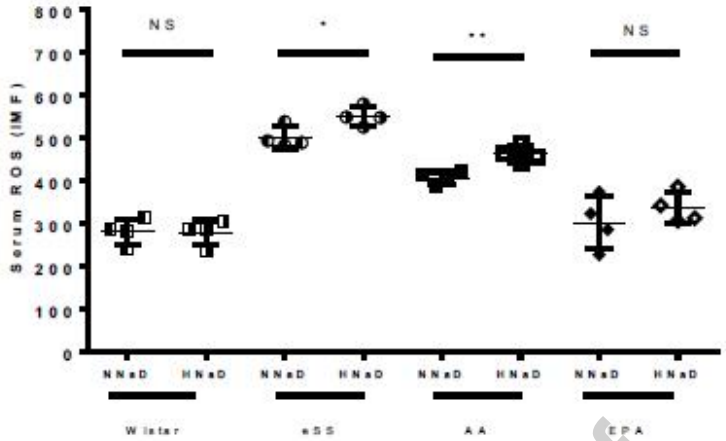



**Figure 2. Serum IL-6 determination before or after HNaD.** Blood samples were drawn from the vein tail and separate serum by centrifugation. After that, serum was maintained to determine IL-6 levels by enzyme-linked immunosorbent assay (ELISA). Dots show IL-6 pg/mL expressed as mean ± SEM, **p ≤ 0.01 and ***p ≤ 0.001 NNaD versus HNaD. t-Test. n = 10.

Fig 3

(a)

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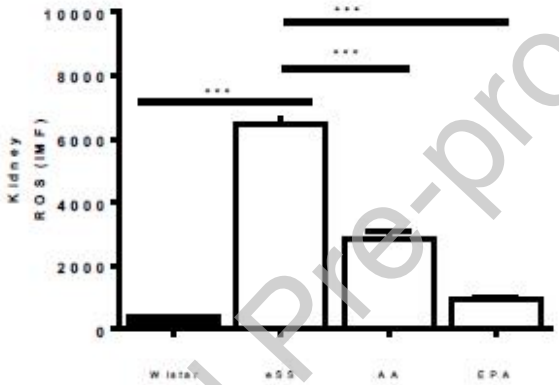


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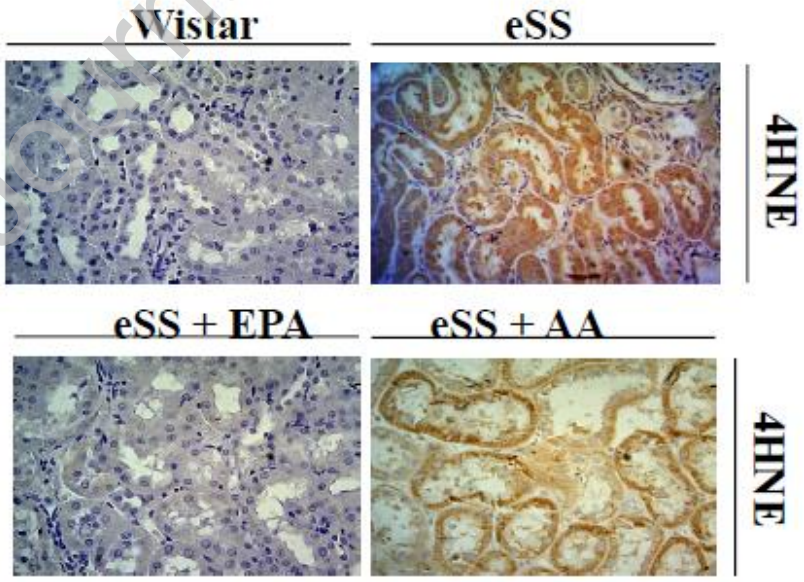

**Figure 3. Intracellular ROS production in T2-DM-bearing rats.** ROS levels were determined by dichlorodihydrofluorescein diacetate fluorescence (IMF) in (a) Kidney tissues lysates after treatment with fatty acids and sodium overload. Bars show IMF expressed as mean ± SEM, ***p ≤ 0.001 eSS versus wistar, AA or EPA. ANOVA. n = 4. (b) Serum from T2-DM-bearing rats before and after a sodium overload. Dots show IMF expressed as mean ± SEM, *p ≤ 0.06 and **p ≤ 0.01 NNaD versus HNaD. t-Test. n = 4.

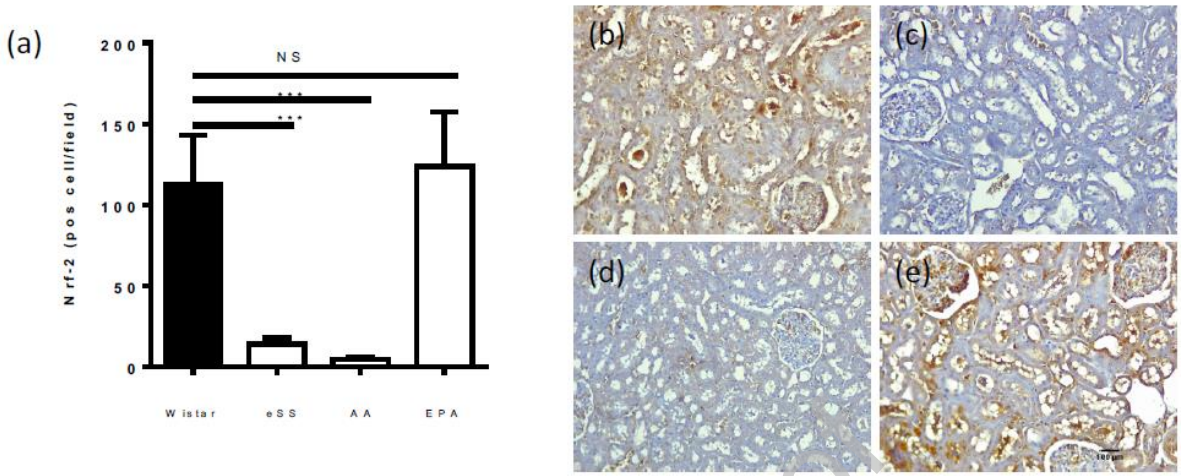


**Figure 4. Nrf2 expression in kidney tissues from** sodium-overloaded, T2-DM-bearing rats after treatment with fatty acids. (a) Immunolabeling for Nrf2 expression in kidney tissues from T2-DM-bearing rats. Bars show Nrf2+ cells/field expressed as mean ± SEM, ***p ≤ 0.001 Wistar versus eSS, AA or EPA. ANOVA. n = 3. Representative images (20× magnification) from (b) wistar, (c) eSS, (d) AA and (e) EPA are shown. Tissues were collected from rats and fixed in paraformaldehyde 4 % for immunostaining. Nrf2+ cells were counted from tissues.

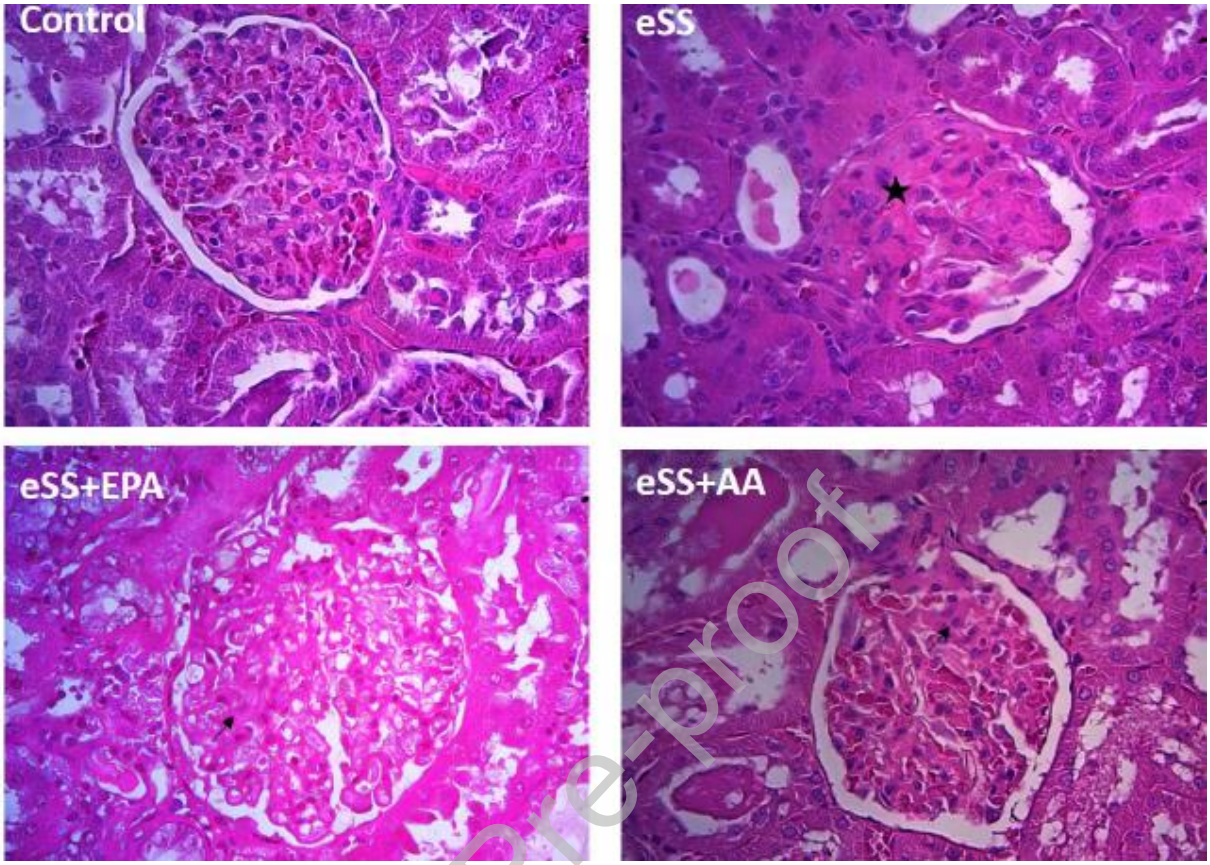


**Figure 5. Representative images of kidneys from T2-DM-bearing.** eSS animals exhbited significant FSGS (asteric) in relation to control rats. EPA and AA treatment induced an important remission of glomerular lesions, showing only mild and segmental mesangial expansion (arrows). Hematoxilin/eosin. Original magnification 400X.

**Table 1. Physical characteristics and biochemistry parameters of the animal groups after NNaD.**

| | Wistar (n=10) | eSS (n=10) | eSS + AA ω-6 (n=10) | eSS + EPA ω-3 (n=10) |
|---|---|---|---|---|
| Weight (g) | 551±4 | 402±22 | 425±26 | 506±20 |
| SBP (mmHg) | 115±6 | 109±6 | 120±4 | 126±2 |
| HbA1c (%) | 4.5±0.1 | 6.43±0.2* | 6.2±0.2* | 6.1±0.2* |
| Postprandial glucose (mg/dL) | 118±1.7 | 136±4.0* | 126±3.6* | 113±2.7*& |
| Serum TAG (mg/dL) | 113±1.5 | 192±11.2* | 160±25.0*# | 141±4.9*# |
| Serum Chol (mg/dL) | 41.2±26.7 | 92.3±2.8* | 61±3.5*# | 68.3±8.6*# |
| Serum Creatinine (mg/dL) | 0.68±0.07 | 0.55±0.02 | 0.62±0.07 | 0.60±0.05 |

3-months-old eSS rats (n=30) were fed with a normal Na-diet (NNaD) (0.4% NaCl) and divided into three groups: i. diabetic control (eSS), ii. eSS treated with arachidonic acid (AA, 20:4 w-6) (AA), iii. eSS treated with EPA (EPA, 20:5 w-3) (EPA), Wistar rats (Wi) were used as healthy controls (n=10). After 1-year treatment with fatty acids, rats were placed for 7 days in metabolic cages and undergone to a high Na-diet (HNaD) (4% NaCl). Before that, body weight (BW), systolic blood pressure (SBP), posprandial glucose (mg/dL) as well as glycosylate hemoglobin (HbA1c), triglycerides (TAG) and cholesterol (Chol) were determined. Values are mean ± SEM (n=10 per group), *p ≤ 0.05 vs Wistar; #p ≤ 0.05 vs ess; & p ≤ 0.05 vs eSS + ω-6.

**Table 2. Morphological changes in kidneys from sodium-overloaded, T2-DM-bearing rats after treatment with fatty acids.**

| | Wistar (n=10) | eSS (n=10) | eSS + AA ω-6 (n=10) | eSS + EPA ω-3 (n=10) |
|---|---|---|---|---|
| Glomerular Sclerosis (0-3) | 0±0 | 0.75±0.64* | 0.01±0.01# | 0±0# |
| Renal interstitial infiltration (0–3) | 0±0 | 1.33±0.33* | 0.67±0.21 | 0.25±0.25# |

Values are mean ± SEM, *p ≤ 0.05 vs Wistar; #p ≤ 0.05 vs eSS.